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NOTES

Sequencing *Bacillus anthracis* Typing Phages Gamma and Cherry Reveals a Common Ancestry

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The genetic relatedness of the *Bacillus anthracis* typing phages Gamma and Cherry was determined by nucleotide sequencing and comparative analysis. The genomes of these two phages were identical except at three variable loci, which showed heterogeneity within individual lysates and among Cherry, Wβ, Fah, and four Gamma bacteriophage sequences.

Bacteriophages are useful tools for bacterial species and strain differentiation (7, 20). Gamma phage (Wγ) susceptibility is an initial test for differentiating *Bacillus anthracis* from closely related *Bacillus cereus* group species (1, 3). Even though lysis is highly specific for *B. anthracis*, there are a few *B. cereus* strains that can be infected by Gamma phage (8, 29). The history of Gamma phage is quite complex. McCloy isolated Wβ phage that was induced from *B. cereus* strain W (ATCC 11950) and found it to be somewhat *B. anthracis* specific, infecting only a few strains of *B. cereus* (21). Wβ formed turbid plaques on *B. anthracis* and failed to infect the original source, *B. cereus* strain W; however, a rare clear plaque mutant, called Wα, could infect both *B. anthracis* and *B. cereus* strain W. Both Wβ and Wα could infect only *B. anthracis* strains that lacked a capsule (21), limiting their usefulness as typing phages. Gamma phage was originally isolated by Brown and Cherry in 1955 as a W phage variant formed by reinfecting *B. cereus* strain W with a lysate of W phage (3). It has the unique properties of being able to infect both smooth (encapsulated) and rough (nonencapsulated) *B. anthracis* strains and being unable to infect *Bacillus* strains that are lysogenic for Wβ phage. Since many *B. anthracis* strains are encapsulated, Wγ became a valuable tool for typing *B. anthracis* strains. Another *B. anthracis* phage called Cherry phage has also been used for typing, albeit less frequently (13); however, its relationship to Gamma phage was not known. Gamma and Cherry phages appear identical under the electron microscope, and both belong to the *Siphoviridae* morphotype (13, 36).

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We completed and analyzed the nucleotide sequences of the Gamma and Cherry phages to determine their genetic relatedness. During the course of sequencing, it was determined through restriction enzyme mapping and PCR experiments that the stock phage preparations were heterogeneous, which led to the acquisition and sequencing of a second Gamma phage preparation from USAMRIID. Comparison of the complete genome sequences has revealed the location of three distinct variable genetic loci. These variable loci were also compared with the sequences of Wβ, $W\gamma^{d}$, $W\gamma^{P}$, and Fah (Table 1). Overall, this work provides a striking example of how diagnostic bacteriophages can evolve over several years in different laboratories.

Bacterial strains and phage DNA isolation. Gamma-LSU phage ($W\gamma^{L}$) and Cherry phage ($W\gamma^{C}$) DNA were provided by Pamala R. Coker while at Louisiana State University. The phages were propagated on *B. anthracis* strain Vollum by plating on Trypticase soy agar with 5% sheep blood (Remel, Kansas) followed by amplification in nutrient broth. Bacterial cells were removed from the lysate by filtration through a 0.22-μm syringe filter prior to isolation of bacteriophage genomic DNA. A stock Gamma-USAMRIID phage ($W\gamma^{U}$) lysate was obtained from John Ezzell, USAMRIID, Fort Detrick, MD, and propagated on *B. cereus* ATCC 4342. A single isolated plaque was picked after overnight growth from a lawn of *B. cereus* ATCC 4342 using the agar layer method (2). Bacteriophages from this plaque were propagated on *B. cereus* ATCC 4342 on agar plates (2). The resulting cell lysate was passed over DE52 cellulose resin to remove unpackaged, contaminating nucleic acids in the lysate (18). The flowthrough was then filtered through a 0.22-μm syringe filter to remove bacterial cells. $W\gamma^{L}$ and $W\gamma^{C}$ genomic DNA was purified using a QIAGEN Lambda DNA extraction kit (QIAGEN, Germany). The DNA extraction procedure was modified from the QIAGEN Lambda DNA extraction kit (QIAGEN, Germany) by resuspending the polyethylene glycol phage pellet in 215 μl of buffer L4 and 4.3

μl of proteinase K (20 mg/ml) followed by incubation at 56°C for 2 h prior to the addition of the remaining buffer L4 and incubation following the manufacturer's instructions.

Genome sequencing and annotation. The complete nucleotide sequences were determined for the *B. anthracis* typing phages $W\gamma^U$ (37,253 bp in length and 35.22% G+C at 34-fold coverage), $W\gamma^L$ (38,067 bp in length and 35.63% G+C at 46-fold coverage), and $W\gamma^C$ (36,615 bp in length and 35.26% G+C at 15-fold coverage) using methods previously described (11). To identify potential coding regions, the Glimmer gene finder (9) was modified by training with a set of *B. cereus* (15, 24, 25) coding regions. Functional assignments for predicted coding regions were based on characterized matches to a nonredundant database and a collection of hidden Markov models (HMMs). Since the sequenced DNA was obtained from functional phages and not bacterial chromosomes, novel nomenclature was used to reflect this. For example, a "hypothetical phage protein" is a coding region that is not similar to anything in the current databases but may be a phage protein. In contrast, a "conserved phage protein" is a coding region that has no defined function but is present in at least one other phage or prophage region. The total numbers of predicted coding regions are 55 ($W\gamma^U$), 50 ($W\gamma^L$), and 51 ($W\gamma^C$). The Gamma and Cherry phages are identical at the nucleotide level except in three loci (Fig. 1 and Table 1). Because these three genomes are so similar, we will refer to them collectively as the Gamma/Cherry phage unless specifying those unique loci. The mean of the BLASTP score ratio (23a) was used to compute best matches. $W\gamma^C$ was the best match to $W\gamma^L$ and $W\gamma^U$, having a BLASTP score ratio close to 1 (perfect match). The next best match is the λBa02 prophage from *B. anthracis* Ames followed by an unpublished induced functional prophage from *Bacillus thuringiensis* 4I1.

Phylogenetic analysis. The gold standard 16S rRNA gene (5, 12, 17) and core housekeeping genes (4, 27) serve as reliable markers for phylogenetic analysis of bacterial and archaeal chromosomal evolution. In contrast, there are no bacteriophage genes that are suitable universal markers for phylogenetic analysis of phage genome evolution (26). Among the tailed bacteriophages (the order *Caudovirales*), there is a relatively conserved operon of genes that code for the proteins responsible for phage packaging and head morphogenesis. Two of these proteins, large terminase (6, 14, 30) and portal (19), have been used in phylogenetic analysis. Recently, the large terminase phylogeny was demonstrated by Casjens and colleagues to have predictive value: the clustering of the large terminase correlates with the mechanism of DNA packaging and with the structure of the virion ends (6).

A phylogenetic tree of large terminase protein sequences was used to determine the group to which the Gamma/Cherry phage belongs (data not shown). The large terminase protein sequences from 32 bacteriophage genomes were aligned using T-Coffee (23). One thousand bootstrapped replicates were generated as described previously (11), except the default settings of the PROTDIST and NEIGHBOR programs were used (10). The large terminase protein of the Gamma/Cherry phage grouped with the phages that generate 3′-extended *cos* ends (data not shown).

TABLE 1. Summary of Gammy/Cherry phage heterogeneous regions

Phage variant	Accession no. and source or reference	Size (bp)	Locus I (bp) and form[a]	Locus II (bp), form, and sequence[c]	Locus III (bp) and function
Gamma-LSU ($W\gamma^L$)	DQ222855; this study	38,067	24591–24592; A†, C*	27018–27056; III; EEEKEEEKEEEEE	30511–33335; collagen superfamily cupin domain protein
Gamma-USAMRIID ($W\gamma^U$)	DQ222853; this study DQ222854; this study	37,253 7,578	24591–25231; B*, C† n.d.	27657–27695; I; KEQKKEQKEEQEE	31150–32512; fosfomycin resistance 3428–6252; collagen superfamily cupin domain protein
Cherry ($W\gamma^C$) Cherry prime ($W\gamma^{C'}$)	DQ222851; this study DQ222852; this study	36,615 6,143	24592–24593; A†, C* 1781–4424; A*	27019–27057; I; KEQKKEQKEEQEE n.d.	30512–31874; fosfomycin resistance Fosfomycin resistance†
Gamma-d'Herelle ($W\gamma^d$) Wbeta (Wβ)	DQ294634; this study DQ289556; 28 DQ289555; 28	674 37,373 40,864	A† 24720–25360; B* 24748–27392; A*	301–336; II; 27786–27825; I; 29818–29857; I; KEQKKEQKEEQEE	Fosfomycin resistance† 31279–32641; fosfomycin resistance 33311–36135; collagen superfamily cupin domain protein
Gamma-Porton ($W\gamma^P$)	DQ221100; unpublished DQ150593; 22	36,083	24594–27238; A*	29664–29700; II; KE-KEKEKQKEEE	Missing bp 28798–31971 (Cherry coordinates)
Fah		37,974	24583–27226; A*	29652–29664; IV; KE-K--------E	32124–33233[b]; cupin domain protein

[a] An asterisk indicates that the form was found in the deposited sequence; a dagger indicates that the form was not found in the deposited sequence but was observed with PCR. n.d., not determined (the locus was not present in the deposited sequence).
[b] Nucleotides 25917 to 32225 of $W\gamma^L$ (GAMMALSU0034 to GAMMALSU0036) are missing.
[c] Underlining indicates conserved amino acid residues.

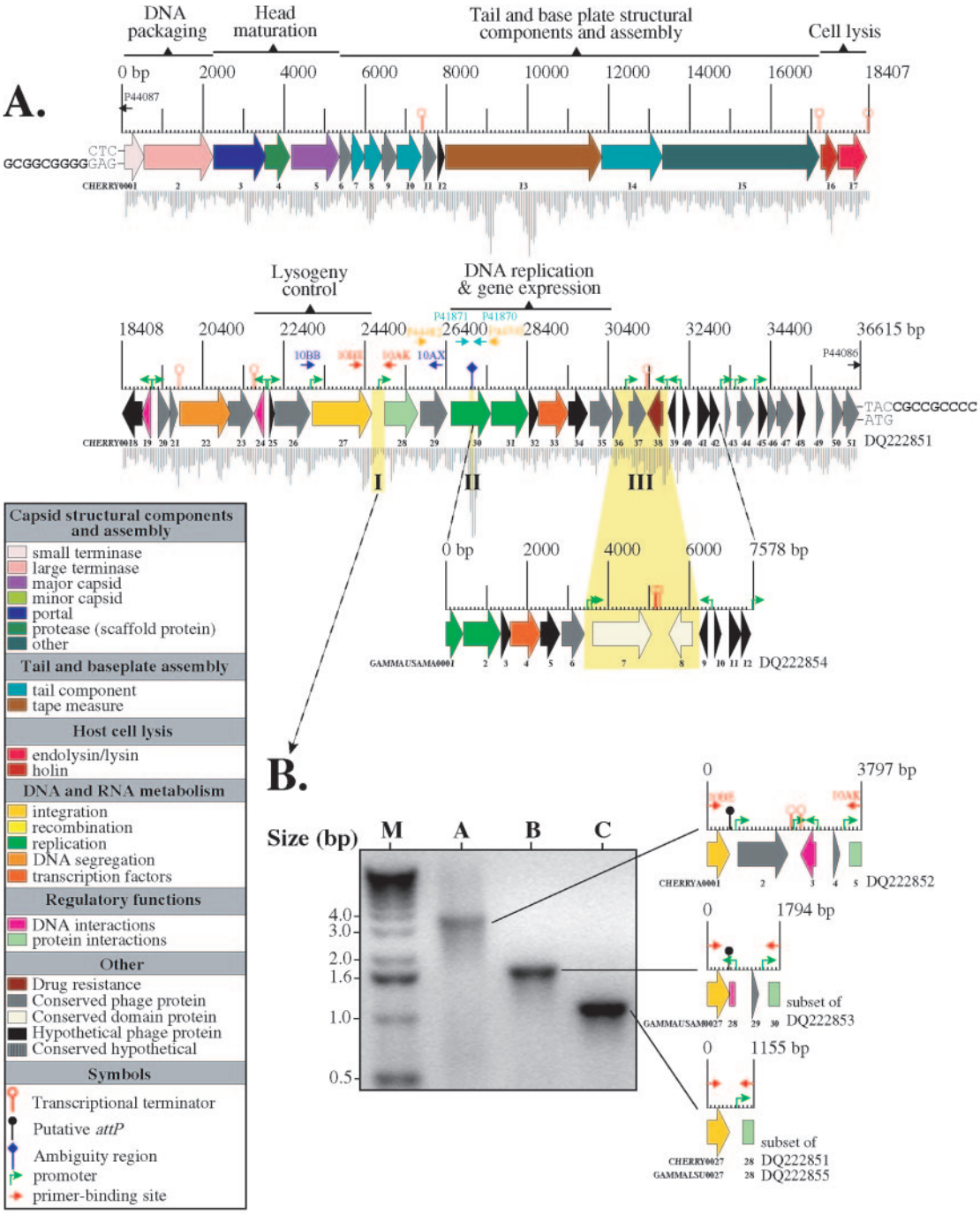

FIG. 1. Linear representation of the Gamma/Cherry phage genomes. (A) A consensus molecule is depicted with the 3′ protruding *cos* ends indicated at the beginning and end of the molecule. The χ^2 value was determined and graphed below the ORFs. Each ORF is color coded based on predicted function. See the key for definitions. Regions highlighted yellow are areas of heterogeneity and are labeled with roman numerals. GenBank accession numbers are indicated to the right of each linear illustration. (B) A digital photograph of an ethidium bromide-stained 1% agarose 1× Tris-acetate-EDTA gel indicates the sizes of the three different forms observed within variable locus I. A 1-kb ladder was loaded into the leftmost lane, followed by forms A, B, and C. The PCR products were obtained through amplification using primers 10BE and 10AK (red arrows). The sequence of form A was determined by cloning and sequencing the PCR product from primers 10BB and 10AX on $W\gamma^{C'}$. Forms B and C were determined from the assemblies of $W\gamma^{U}$ and $W\gamma^{C}$, respectively.

Gamma and Cherry phages package DNA using a 3′ overhang *cos* site mechanism. Since the large terminase protein of Gamma and Cherry phages grouped with phages of gram-positive bacteria having known 3′ overhang single-stranded cohesive (*cos*) ends and we observed no terminal redundancy in the genome sequence, which would have suggested a *pac* site mechanism of DNA packaging, we hypothesized that the Gamma/Cherry phage packages DNA using a 3′ overhang *cos* site mechanism. We tested this hypothesis by sequencing the PCR product that was formed after religation of the *cos* ends (Fig.

1). Two unique primers, P44087 (TCAATCTGACTAATTCA GCAGC) and P44086 (GGATAAGAATAGATACTACGA CC), were designed to face outward and read the DNA sequence of each end of the linear phage genomic DNA (Fig. 1). By comparing the sequence of the PCR product to the sequence of the ends, the sequence of the *cos* site, CGCCG CCCC (Fig. 1A), was determined to be 9 nucleotides in length, which is similar to the *cos* site of related *Clostridium perfringens* phage ϕ3626 (CGCAGTGTC) and identical to the *cos* site of *B. anthracis* bacteriophage Fah (22).

Regions of heterogeneity. We identified three heterogeneous loci while comparing the sequences of the Gamma and Cherry phages (Fig. 1A, yellow highlighted areas). We first became aware of heterogeneity near the integrase when performing confirmatory restriction mapping of the Wγ^{C} genome from a plaque-purified phage preparation grown on *B. cereus* ATCC 4342 (Table 1, locus I). The map revealed additional DNA that was not included in the Cherry phage assembly (data not shown). Primers 10BB (AATTGTATCATCGAGTATTAAT AGC) and 10AX (TGTAAGTATCGATACCTAATCG) were designed to subclone this conflicting region using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), for the production of a microlibrary for sequencing and for primer walking of the PCR product. For diagnostic purposes, primers 10BE (TGTGGTG AGCCAATTACAGC) and 10AK (TTTCGCTATCTGCATA TTTGAG) were designed to amplify this locus (Fig. 1B). PCR using primers 10BE and 10AK generated a 1,155-bp product (form C) for Wγ^{C} and Wγ^{L} but a 3,797-bp product (form A) for this variant, which we refer to as W$\gamma^{C'}$ (Cherry prime; DQ222852) (Table 1). Assembly of the previous Cherry sequences with the sequence of the 3,797-bp PCR product reconciled the restriction map data.

When a different stock of the Gamma phage (Wγ^{U}) was sequenced, we found that this region turned out to have yet another form, with a size of 1,794 bp (Fig. 1B, form B). To determine the scope of variability in this region, we conducted PCR experiments with primers 10BB and 10AK on 24 well-isolated plaques from each stock lysate grown on *B. cereus* ATCC 4342 (data not shown). From these results, we concluded that there were three distinct forms (A, B, and C) from this region of the Gamma/Cherry phage genomes and that each stock tested is not genetically pure. For the Wγ^{U} stock, there were 13 out of 23 total plaques (57%) that were positive for form B (1,794 bp) and 10 out of 23 (43%) that had form C (1,155 bp), but there was no PCR product for form A. Wγ^{L} contained form A (3,797 bp) in 16 out of 20 (80%) and form C in 5 out of 20 (25%) of those plaques that gave a product but no form B. Wγ^{C} was similar to Wγ^{L} in that no form B was observed, but 14 out of 21 plaques (67%) amplified the form A product and 7 out of 21 (33%) gave form C. Only Wγ^{U} produced form B.

The second locus of heterogeneity was initially discovered only in the Wγ^{C} preparation, affecting the coding sequence of a putative replisome organizer (CHERRY0030; Table 1, locus II, and Fig. 1A, blue diamond). At coordinates 27025 to 27049, the consensus sequence of Wγ^{C} from the whole shotgun assembly was (STTcttyTTKgTTKTTCTTTTTYTTK; lowercase letters indicate the presence of gaps in some of the aligned sequences). Further inspection of the underlying sequence reads showed that this ambiguous sequence was the result of a composite of two distinct sequences, each having about equal numbers of supporting clones. There were two library clones that matched part of the form A sequence and bridged the ambiguity region, which provided assembly data to support two distinct forms near the integrase. To determine whether form I or II sequences belonged with the Wγ^{C} or W$\gamma^{C'}$ phages, we designed nested sets of the primers P44705 (TGATTTTCTA TGATGCTGTGTTG) and P44482 (AATAGTTGAAGAAT ATACACTTCC) to first amplify a 2,165-bp product and then primers P41871 (CCCATACAACTCAATTGGGAG) and P41870 (GTGCAAATAACGTGCTCGGTC) to obtain high-quality sequence data close to the ambiguity region (Fig. 1). The sequences of these PCR products confirmed that the form II ambiguity sequence is linked to form A (W$\gamma^{C'}$) and the form I ambiguity sequence is linked to form C (Wγ^{C}). Since this study was completed, the sequences of two additional Gamma phage isolates (Wγ^{d} [28] and Wγ^{P} [unpublished]), Wβ (28), and Fah (22) have become available for comparison. With the addition of Fah, this locus was expanded to 13 amino acids, with a total of four different variations observed (Table 1).

A third locus of heterogeneity between Gamma and Cherry phages was identified during comparative analysis of the three phage genomes (Table 1 and Fig. 1A, locus III). Wγ^{U} and Wγ^{C} main assemblies have identical sequences in this region, while Wγ^{L} and a 7,578-bp variant assembly from Wγ^{U} (Fig. 1A) share a different sequence. This region in Wγ^{U}/Wγ^{C} encodes three proteins (GAMMAUSAM0038/CHERRY0036, GAMMAUSAM0039/CHERRY0037, and GAMMAUSAM0040/CHERRY0038). Both GAMMAUSAM0038/CHERRY0036 and GAMMAUSAM0039/CHERRY0037 have matches to proteins with no known function from other phages. GAMMAUSAM0040/CHERRY0038 is predicted to encode a fosfomycin resistance protein (Table 1). It is unclear whether GAMMAUSAM0040/CHERRY0038 is able to produce a functional protein, because the insertion of a cytosine nucleotide at position 67 caused a frameshift in both Wγ^{U} and Wγ^{C}; however, a nonframeshifted homolog, *gp41* in Wγ^{d}, was recently shown to confer fosfomycin resistance (28).

The equivalent region in Wγ^{L} and a 7,578-bp assembly from Wγ^{U} (Fig. 1) is larger than the region in the Wγ^{C} and Wγ^{U} main assembly, encoding two proteins (GAMMALSU0036/GAMMAUSAMA0007 and GAMMALSU0037/GAMMAUSAMA0008). GAMMALSU0036/GAMMAUSAMA0007 is predicted to encode a 479-amino-acid protein with 95 copies of a G-X-X repeat that is found in members of the collagen superfamily and proteins that are structural components of the exosporium of *B. anthracis* (33) and *B. cereus* (35) spores and form a triple helix. The distribution of repeats has the structure $[GXX]_5$-T-$[GXX]_{43}$-P-$[GXX]_5$-P-$[GXX]_4$-T-$[GXX]_{38}$. This open reading frame (ORF) is predicted to belong to the collagen repeat superfamily based on HMM (PF01391) and BLASTP matches. GAMMALSU0037/GAMMAUSAMA0008 is predicted to encode a 193-amino-acid protein that matches HMM PF07883, a cupin domain protein. In bacteria, proteins with one or two cupin domains, which form a beta barrel structure, can have either isomerase or epimerase activities that modify cell wall carbohydrates. The best NCBI-BLASTP match is a hypothetical protein, CTC01899 from *Clostridium tetani* E88.

We propose that the Gamma phage encodes the collagen repeat protein either to function in host recognition or possibly

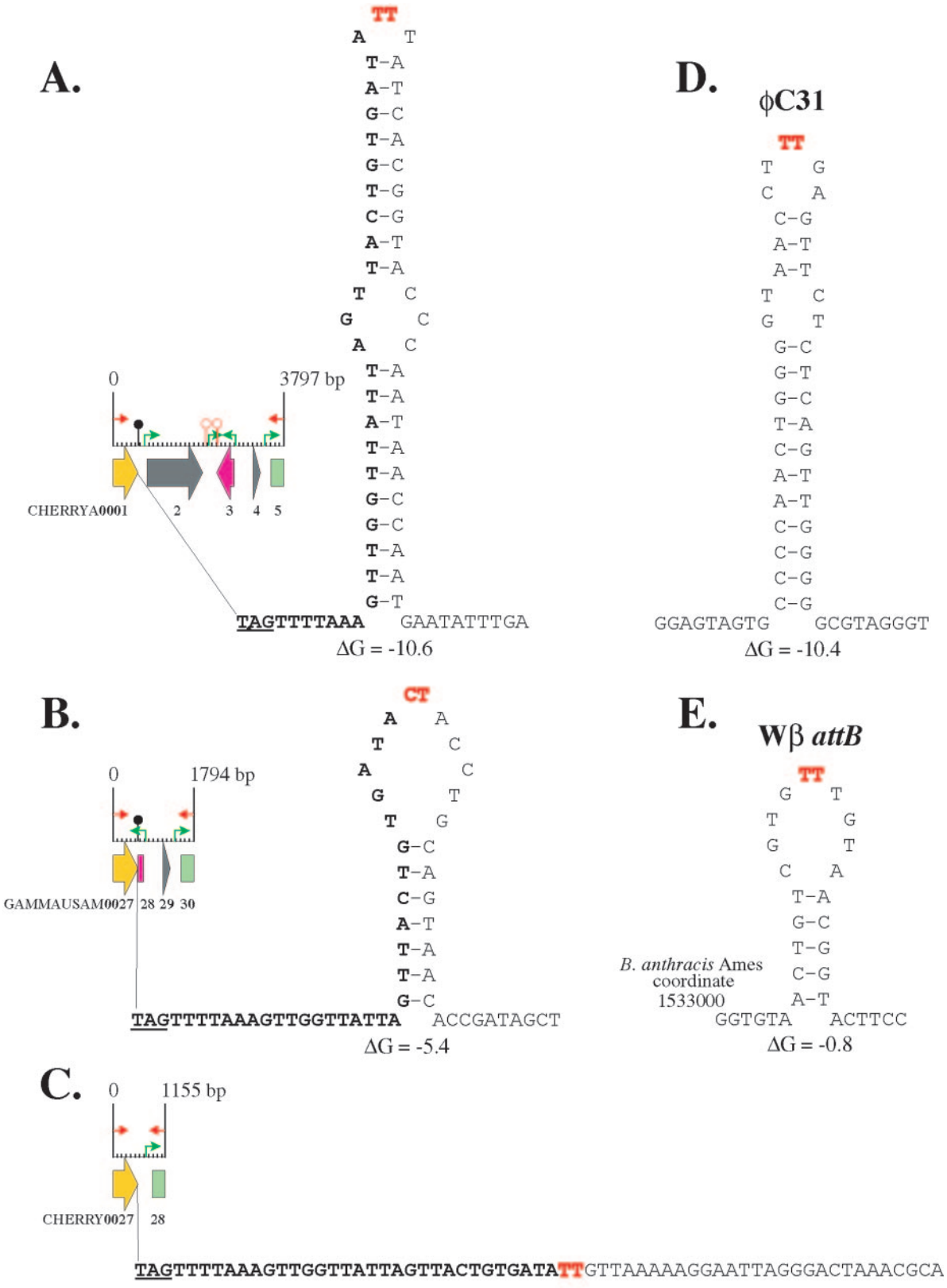

FIG. 2. Putative *attP* of Gamma and Cherry phages. Predicted *attP* sites are depicted as stem-loop structures when present (A and B). The linear representations of $W\gamma^{C'}$ (A), $W\gamma^{U}$ (B), and $W\gamma^{C}/W\gamma^{L}$ (C) are color coded as in Fig. 1. The known *attP* site from φC31 (31) is provided for reference (D). The predicted structure of *attB* within BA1618 of *B. anthracis* is indicated (E) (R. Calendar, personal communication). Stem-loop structures aid in visualization of the inverted repeats that flank the core *att* sequence (red bold). Predicted secondary structures and their free energies are from MFOLD. The stop codon of the phage integrase gene is underscored, while the boldface type denotes the common sequence 5′ of the breakpoint of each of the three forms observed.

to make the bacillus spore more stable, ensuring its survival under stress. It is also entirely possible that either the collagen repeat protein or the cupin domain protein or both account for the ability of bacteriophage Gamma to infect encapsulated *B. anthracis* strains when Wβ cannot. The Gamma phage has not been shown to form lysogens in *B. anthracis*, but the allelic variant Wα has been shown to survive within *B. anthracis* spores (16). This phage-trapping phenomenon has been observed during infection of *B. subtilis* 3610 by the virulent phage Φe (32) and by phage PBS1 in *B. subtilis* SB19 (34).

There is also the question of the origin of fosfomycin resistance and the collagen repeat/cupin domain regions. It is possible that through propagation of these phages on various hosts, in various labs, they acquired these loci via recombination with prophages that existed in the host genome. We have evidence that contradicts this hypothesis, because PCRs on *B. cereus* strain W and on a mitomycin-induced prophage from strain W (presumably Wβ) gave products for both regions (data not shown). This indicates that these two forms existed in the parental host strain W.

The Gamma/Cherry phage is predicted to encode serine recombinases. The type of recombinase encoded by a bacteriophage determines target site specificity. For example, tyrosine recombinases that have a tropism for tRNA genes typically have what appears to be a target site duplication flanking the ends of the integrated prophage genome, which corresponds to the core sequence of the *att* site. In contrast, serine recombinases have very small core *att* sites that are flanked by inverted repeats (31) and may or may not have any recognizable target site duplication. An in silico method to identify the type of recombinase is to use HMMs. GAMMAUSAM0027 of $W\gamma^U$, GAMMALSU0027 of $W\gamma^L$, and CHERRY0027 of $W\gamma^C$ match PF0235, an HMM model for serine recombinases, above the trusted cutoff. Multiple sequence alignments of the Gamma/Cherry recombinases with members of the serine recombinase family (data not shown) enabled a prediction of the catalytic serine residue at amino acid residue 13.

Nucleotide sequence and structure of a putative *attP* site. Sequence analysis of the three forms (A, B, and C) near the integrase *attP* region of the Gamma/Cherry phage revealed a conserved breaking point 31 nucleotides downstream of the integrase stop codon (Fig. 2A, yellow ORF). Further inspection of this region revealed inverted repeats with the breakpoint in the center of predicted stem-loop structures (Fig. 2). It is common for serine recombinases to use inverted repeats as the substrate for integration (31). The MFOLD program (37) was used to calculate the structure and free energy of the putative *attP* region for two of the three forms (Fig. 1 and 2). The largest *attP* region (Fig. 1B and 2A, form A) was predicted to form the best inverted repeat and the most stable structure ($\Delta G = -10.6$ kcal). The medium-sized *attP* region (Fig. 1B and 2B, form B) formed a stem-loop with predicted free energy of −5.4 kcal (Fig. 2). We were unable to find an inverted repeat or a predicted secondary structure for the smallest *attP* region (Fig. 1B and 2C, form C), suggesting that this form may have been derived through illegitimate recombination. Given these data, we hypothesize that form C is unable to integrate into *attB*, while forms A and B may be functional *attP* substrates capable of site-specific integration into *attB*. Form A is the ancestral form, since Wβ has this sequence (Table 1). Form A was shown to serve as a substrate for site-specific recombination in *B. anthracis* by targeting BA1618 (Fig. 2) (R. Calendar, personal communication). Bacteriophages Fah, $W\gamma^{C'}$, and $W\gamma^P$ also have form A (Table 1), suggesting that these phages are also capable of integration/excision reactions. Further studies are necessary to determine whether form B is a functional *attP* sequence.

We present the complete nucleotide sequences and a comparison of two *B. anthracis*-specific bacteriophages that are used for typing. To our surprise, we discovered heterogeneity within each of three phage lysate stocks used to make purified phage DNA for whole shotgun sequencing. There was also heterogeneity between Gamma phage stock lysates from two different sources and among Wβ, $W\gamma^d$, $W\gamma^P$, and Fah sequences. We conclude that the Gamma phage, Cherry phage, and Fah are essentially the same phage, containing variations at three distinct locations within the genome and demonstrating significant heterogeneity within their populations.

Nucleotide sequence accession numbers. The nucleotide sequences of *B. anthracis* $W\gamma^L$, $W\gamma^U$, and $W\gamma^C$ genomes and minor variant assemblies have been deposited at GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) under accession numbers DQ222851 to DQ222855 and DQ294634.

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